PCT/EP2004/009242

11.10.04

INVESTOR IN PEOPLE

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I also certify that the attached copy of the request for grant of a Patent (Form 1/77) bears a correction, effected by this office, following a request by the applicant and agreed to by the Comptroller-General.

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Signed R. Mahoney

Dated 24 September 2004

PCT/EP2004/009242

11.10.04

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Request for grant of a patent

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Cardiff Road
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NP9 1RH

1. Your reference — P20052GB

2. Patent application number *(The Patent Office will fill in this part)* — 0315512.4

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

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~~UK~~

03JUL03 E819711-3 D00032
P01/7700 0.00-0315512.4

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Patents ADP number *(if you know it)* 87097680001

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

Diagnostic Test

5. Name of your agent *(if you have one)* — ELKINGTON AND FIFE

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

ELKINGTON AND FIFE
PROSPECT HOUSE
8 PEMBROKE ROAD
SEVENOAKS
KENT, TN13 1XR

Patents ADP number *(if you know it)* 67004

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number	Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
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See note (d))

No

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Continuation sheets of this form	
Description	6
Claim(s)	1
Abstract	
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10. If you are also filing any of the following, state how many against each item.

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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	1
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature G Wright. Date 02.07.03

12. Name and daytime telephone number of person to contact in the United Kingdom: Dr Gordon Wright – 01732 458881

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Diagnostic Test

This invention relates to a diagnostic test for the detection of food intolerance.

The terms food intolerance, food sensitivity or hypersensitivity and food allergy are often used loosely and interchangeably to describe any clinically abnormal response attributed to an exposure to a food or food component. This ill-defined, confused terminology is most probably due to a lack of understanding of the pathogenesis of the condition, in turn resulting in the lack of proper diagnostic procedures. However, all terms imply adverse reactions to foods, whether immunological or non-immunological.

In contrast, the term food allergy is restricted to clear, immediate (within one hour) IgE-dependent reactions (Type I) of the adaptive immune system. Upon encounter of a specific allergen to which there has been prior exposure, IgE sensitised mast cells (and basophils) release mediators that trigger the acute inflammatory response. In some individuals, life threatening anaphylactic shock can result. Diagnosis is generally supported by a positive skin prick test or a radio-allergosorbent test (RAST).

Food intolerance however is a slow, chronic inflammatory response to a food that is eaten regularly or in great quantity. The onset symptoms are delayed up to 48 and sometimes 72 hours or more after eating the food. Its mechanism is not fully understood. It may involve IgG, IgM, IgA antibodies and cellular-mediated responses (type II, III & IV) together with complement and features of the innate (non-adaptive) immune system such as those mediated by lectins. There will also be some overlap with symptoms of Type 1 hypersensitivity where mast cell degranulation can occur with Type II hypersensitivity through complement activation. In addition, inflammation resulting from food intolerance can also be due to either toxic effects of constituents in food, (eg. lectins again, salicylates, food colourings, amines, etc.) or to inborn or acquired errors in metabolism as seen in the example of lactose intolerance. Food intolerance is rarely diagnosed by skin tests. Yet, it may be an underlying mechanism contributing to some serious chronic and even autoimmune diseases. Furthermore, several studies have shown that many conditions such as migraine, irritable bowel

syndrome, rheumatoid arthritis, respond to dietary modification. However, the offending foods were not identifiable by skin prick test or RAST.

One method of assaying whole blood is the antigen leukocyte cellular antibody test (ALCAT), which is intended for the determination of the effects of multipathogenic mechanisms involved in food sensitivities. Reactions of aliquots of whole blood with individual food extracts are carried out under carefully controlled conditions and compared with control aliquots identically treated but not exposed to the food extracts. After separation of the white cell fraction, each aliquot is analysed using a specially modified cell sizer and counter capable of separating the white cells into hundreds of subsets based on cell size.

The analyzer measures cell number, mean cell size and size distribution curves. The data is analyzed and positive reactions are indicated by a change in cell size and/or absolute loss of cells. Variants of this test have been developed in which the electrical properties of the white blood cells are investigated. A significant shift from control values is held to signify clinically relevant food intolerance.

Both of these methods suffer from the disadvantage that they require costly and specialised equipment to carry out the analysis of food intolerance. In addition, these test procedures require relatively large samples of whole blood to be drawn from the subject, eg between 15 and 20 mL.

An alternative approach is to detect indirectly the inflammatory response to food sensitivity by measuring substances or markers that reflect its different aspects. One method is confined to measuring IgG, (the Yorke Test) based on the assumption that food intolerance is exclusively a reaction due to the adaptive immune system. Other methods measure the release of chemical mediators. One such example is the Food Allergen Cellular Test (FACT), where food sensitised leucocytes release leukotrienes which it is claimed reflect both IgE dependent and independent allergic responses but are unable to distinguish between food allergy and intolerance.

Many of the prior art methods of diagnosing food intolerance are indirect methods which do not address directly the activation of neutrophils in the blood by certain foods.

Surprisingly, we have found a new diagnostic method, which overcomes or substantially mitigates the disadvantages of prior art methods, whilst at the same time giving reliable measures of food intolerance by directly measuring neutrophil activation on exposure to foods.

According to the invention we provide a method of diagnosing intolerance to a specified food by a subject which comprises incubating a leukocyte sample prepared from whole blood drawn from the subject with an extract of said food and detecting whether or not neutrophils in the sample have become activated.

A number of methods can be used to determine if neutrophils have become activated. If desired, the change in neutrophil morphology which results on activation can be determined optically, eg using a microscope. However, although this method is accurate, it is very time consuming. Alternative methods of detecting neutrophil activation include luminescence, detecting the uptake of dyes or fluorescent markers, photometry, detecting release of cytokines, detecting release of bioactive molecules (eg leukotrienes), microbial proteins (eg defensins) and/or free radicals (eg reactive oxygen intermediates).

A particularly preferred method makes use of the fact that neutrophils tend to be become more adhesive on activation. As such, a preferred method of detecting neutrophil activation is to detect the increased adhesiveness of an activated neutrophil, relative to an unactivated neutrophil. In particular, activated neutrophils tend to adhere to plastics surfaces, eg the surface of a well in a 96 well titre plate or in a sample tube, for example an Eppendorf® tube. Accordingly, neutrophils adhering to a plastics surface can be used as a measure of neutrophil activation.

In general, when the neutrophil adhesiveness is determined by detecting adhesion to a plastics surface, such as the well of 96 well plate or an Eppendorf® tube, non-adhering

cells are removed by washing. The neutrophils adhering to the plastics surface can be assayed by a number of method, eg those methods mentioned above. When the surface is a well, eg in a 96 well titre plate, the well can be scored turbidometrically. However, preferably, adhesion is detected by lysis and assaying for one or more intracellular markers of neutrophil activation. Methods of cell lysis are well known in the art and include washing adhered cells with detergent, eg Triton X100. Suitable markers for neutrophil activation include acid hydrolases, myeloperoxidases, lysozyme, lactoferrin, neutral proteinases and serine proteinases. Markers that may be specifically mentioned include SGOT (serum glutamic oxaloacetic transaminase), SGPT (serum glutamic pyruvic transaminase) and particularly LDH (lactic dehydrogenase).

LDH is particularly preferred as a marker of neutrophil adhesiveness, as the ratio of intracellular to extracellular enzyme in the activated neutrophil is particularly high, eg of the order 1:500.

The method is preferably carried out using a 96 well titre plate, allowing an array of food extracts to be investigated in run, together with suitable controls.

Example

1. Preparation of Food Extracts

Freeze dried food extracts are available commercially and were stored at -20°C before use. When required, extracts are allowed to come to room temperature and then weighed out into 50mL plastic centrifuge tubes. In general 0.1 to 2g of food extracts are weighed out.

20mL of 0.2% w/v phenol in saline (0.9 w/v sodium chloride) is added to each and thoroughly mixed. Tubes are capped and subjected to rotary mixing for 60 hours at room temperature, followed by 20 minute centrifugation at 4500 rpm at 4°C. The supernatant is now filtered under sterile conditions, firstly through a 30-50μm pore size and then through 0.2-0.3μm bacterial filters. The resulting filtrate constitutes the stock solution and can be used for up to one month when stored at 2-8°C. Appropriate

aliquots of stock are then diluted 1:21 with 0.9%w/v saline to produce working solutions ready for use in the diagnostic test. These solutions are stable at 2-8°C for two days.

2. Preparing the sample of leukocytes

A sample of 2ml of whole blood is drawn from subjects being tested by phlebotomy into CPDA or ACD blood tubes with anticoagulant. An aliquot of 270µL Plasma Gel® is added followed by gently mixing for 1min. Tubes are tilted at an angle of 45° and left for 1 hour in this position. The erythrocytes (red blood cells) sediment during this time leaving the white blood cell fraction in plasma which now constitutes the supernatant. For optical microscopy counting, a 10µL aliquot of supernatant is removed and mixed gently by inversion with 490µL of toluidine blue solution in a small plastic tube. A microscopic haemocytometer slide is prepared and the dyed cells are introduced and the liquid volume fixed by placement of a cover slip. Cells are then identified into respective types and counted.

For other methods of assessing activation, physiological saline is incubated to 37°C during the sedimentation with Plasma Gel. An optional volume, eg 1.0 ml, of supernatant is removed and diluted (approximately 1:50) with the saline ready for the food challenge step. The dilution of cells gives an average of 40,000 (40K) between ranges of 15K-80K which are suitable to provide adequate responses in subsequent assays.

3. Reacting the cells with a library of food extracts.

100µL aliquots of a library of 96 different food extracts, (including blanks), are dispensed into a standard 96 well microtitre plate, (polystyrene) from working dilutions prepared fresh each day from stock solutions previously prepared according to conventional methods. The exact method of food extract preparation (...) and the concentration of extract used, will depend, to some extent on the protocols followed by the person or laboratory carrying out the test. However, in general, the food extracts are

prepared from raw, whole foodstuffs which are prepared as diluted immediately prior to use.

Sample aliquots (100μL) of supernatant are then added to each well followed by gently mixing for 1 min. Plates are then centrifuged at 700rpm, for 4 min, then incubated for 1 hour at 37°C.

Each plate is manually inverted over a sink, tapped vigorously and blotted onto tissue paper, then washed twice with saline in order to remove unbound cells. Aliquots of 100μL of 1% Triton X100 detergent are then added to each well and mixed for 2 min.

4. Assay for adherence of activated neutrophils.

Neutrophil activation is measured by assaying for lactate dehyrogenase (LDH) using conventional methods known per se. For example, LDH developing reagent (100μL), is added to each well followed by 30 min incubation, with the plates kept in darkness. The developed colour is then read in a plate reader and absorbances recorded for a bichromatic read at 492 and 620nm. A suitable LDH reagent is that obtainable from Roche as the 'Cytotoxicity Detection Kit Cat No 1 644 793.

Reactive foods that induce food intolerance are identified as those that give a statistically significant response above the baseline response.

Results are obtained by the following procedure; Absorbance differences are calculated for each food extract between spectrophotometric reads at 492nm subtracted from the reference read at 620nm. This is termed the response. The same is repeated for the control population, (n=12), to determine a baseline response from which a significance level at 2.5 standard deviations is calculated. Outlier rejection in the controls is set at 100mAU and if more than 3 outliers occur then the run is rejected. A list of food extracts is compiled in ranked descending order of the absorbance response. Foods observed to be above the level of significance are identified as those causing food intolerance. These are listed in descending order of magnitude and constitute the result. Non-reactive foods are listed separately.

Claims

1. A method of diagnosing intolerance to a specified food by a subject which comprises incubating a leukocyte sample prepared from whole blood drawn from the subject with an extract of said food and detecting whether or not neutrophils in the sample have become activated.

2. A method according to Claim 1, wherein neutrophil acitivation is detected optically.

3. A method according to Claim 1, wherein the neutrophil activation is detected by luminescence, detecting the uptake of dyes or fluorescent markers, photometry, detecting release of cytokines or by detecting release of bioactive molecules, microbial proteins and/or free radicals.

4. A method according to Claim 1, wherein neutrophil activation is detected by determining whether or not neutrophils in the sample have become adhesive.

5. A method according to claim 4, wherein neutrophil adhesion is determined by assaying adhesion to a plastics surface.

6. A method according to Claim 5, wherein the plastics surface is a plastics multi-well titre plate.

7. A method according to Claim 6, wherein the titre plate contains 96 wells.

8. A method according to Claim 4, wherein adhesion is detected by lysis and assaying for one or more intracellular markers for neutrophils.

9. A method according to Claim 8, wherein the one or more markers are selected from acid hydrolases, myeloperoxidases, lysozyme, lactoferrin, neutral proteinases and serine proteinases.

10. A method according to Claim 9, wherein the one or more markers are selected from serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase and lactic dehydrogenase.

11. A method according to Claim 10, wherein the marker is lactate dehydrogenase.

12. A method according to Claim 5, wherein the plastics surface is washed to remove unreacted cells, after incubation of the sample with the food extract but before assaying adhesion.